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Comparison of [^{3}H]Oestradiol and [^{125}I]Oestradiol as Ligands for Oestrogen Receptor Determination

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Summary: The newly available ligand [16α-^{125}I]3,17β-oestradiol was investigated for its suitability for routine oestrogen receptor determinations. Unlabeled 16α-iodo-oestradiol was synthesized. No difference in the relative binding affinity to human uterine oestrogen receptor could be demonstrated, but unlabeled as well as labeled 16α-iodo-oestradiol do not bind to serum sex hormone binding globulin. At 0–4 °C [^{3}H]oestradiol reaches, when incubated with oestrogen receptor its equilibrium after 3 h, [^{125}I]oestradiol after 9 h.

In 100 human mammary tumour cytosols oestrogen receptor concentrations were determined using either [^{3}H]oestradiol or [^{125}I]oestradiol. The mean K_d-values obtained were 0.5 ± 0.3 nmol/l using [^{3}H]oestradiol and 0.42 ± 0.25 nmol/l for [^{125}I]oestradiol. For the linear regression (independent variable [^{3}H]oestradiol, dependent variable [^{125}I]oestradiol) the following sample estimates were obtained: n = 100, r = 0.982, y_i = 3.04 + 1.00035 x. For a 0.95 probability of confidence it can be stated that the sample estimates are indeed comparable according to the criteria of the two ligands.

Vergleich von [^{3}H]Östradiol und [^{125}I]Östradiol für die Bestimmung von Östrogenrezeptoren

Zusammenfassung: [16α-^{125}I]3,17-Östradiol wurde auf seine Brauchbarkeit zur Bestimmung von Östrogenrezeptoren mit dem bisher verwandten [^{3}H]Östradiol verglichen. Unmarkiertes 16α-I-3,17β-Östradiol wurde synthetisiert. Es ergab sich kein Unterschied in der relativen Bindungsaffinität zwischen Östradiol und 16α-I-Östradiol am Östrogenrezeptor. 16α-I-Östradiol zeigte aber keinerlei Bindung zum Sexhormon-bindenden Globulin im Schwangerenserum. Wurde [^{3}H]Östradiol mit Östrogenrezeptor bei 0–4 °C inkubiert, so wurde das Gleichgewicht nach 3 h erreicht. [16α-I]Östradiol benötigte für die Gleichgewichtseinstellung 9 h.

In Cytosol-Präparationen von 100 menschlichen Mammakarzinomen wurden die Östrogenrezeptor-Konzentrationen sowohl mit [^{3}H]Östradiol als auch mit [^{125}I]Östradiol bestimmt. Die ermittelten K_d-Werte waren 0,5 ± 0,3 nmol/l für [^{3}H]Östradiol und 0,42 ± 0,25 nmol/l für [^{125}I]Östradiol. Die mit den beiden Liganden erhaltenen Östrogenrezeptor-Konzentrationen wurden miteinander verglichen: n = 100, r = 0,982; y_i = 3,04 + 1,00035 x.

Introduction

The oestrogen receptor content of a human mammary tumour has proved to be a valuable parameter for the selection of a patient's therapy (hormone or chemotherapy). Approximately 68% of all human breast tumours contain significant amounts of oestrogen receptor (< 10 fmol/mg cytosol protein). 65% of patients with oestrogen receptor-positive tumours respond to hormonal manipulation (1–7). Additional information for prediction of response to hormone therapy can be obtained by determination of progesterone receptors. On the "Consensus Meeting on Steroid Receptors in Breast Cancer" (8) the dextran coated charcoal assay (9) and the sucrose density gradient analysis (10) were generally accepted as ideal routine methods for steroid receptor determinations.

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Recently, a radio-iodinated derivative of oestradiol was synthesized (11, 12). Initial studies indicated that its performance in the oestrogen receptor assay is equivalent to that of tritium-labeled oestradiol (13, 14). In the present study the binding characteristics of commercially available [16α-^{125}I]3,17β-oestradiol and of [^{3}H]3,17β-oestradiol were investigated. We compared the binding of the two ligands to human sex hormone binding globulin, the rate of association with human uterine oestrogen receptor, the binding specificity and oestradiol receptor concentrations in 100 human mammary tumour cytosols.

Materials and Methods

Reagents

[2,4,6,7-^{3}H(N)]3,17β-oestradiol (spec. activity 3.74 TBq/mmol = 101 Ci/mmol), [16α-^{125}I]3,17β-oestradiol (spec. activity 8.89 – 11.11 TBq/mmol = 240 – 300 Ci/mmol), [^{3}H]dihydrotestosterone (spec. activity 1.87 TBq/mmol = 50.6 Ci/mmol), the ^{125}I and the tritium reference sources for calibration of the scintillation counter were purchased from New England Nuclear (Boston, MA 02118). Unlabeled steroids as well as diethylstilboestrol were purchased from Serva (Heidelberg, F.R.G.). All other chemicals were of reagent grade. Scintillation cocktail was from Zinsser (Heidelberg, F.R.G.). Protein standard was from BIO-RAD Laboratories (Richmond, CA 94804).

Assay buffer

10 mmol/l K_2HPO_4, 10 mmol/l KH_2PO_4, 1.5 mmol/l disodium-ethylene-diaminetetraacetate, 3 mmol/l sodium azide, 100 g/l glycerol, pH 7.5. Dextran-coated charcoal suspension: Norit A, 5g/l, Dextran T-500 0.5 g/l in assay buffer, pH 7.5.

Preparation of cytosol

The tissues were pulverized in a micro dismembrator (Braun Melsungen), extracted with assay buffer and centrifuged for 30 min at 105000 *g*. The supernatant was taken as cytosol.

Oestrogen receptor control powder

An oestrogen receptor control powder was purchased from New England Nuclear. It contained 76 ± 24 fmol/mg protein as determined by the manufacturer. The lyophilized animal tissue powder was suspended in assay buffer and centrifuged as described above. These reference powders fulfill the criteria of American quality control.

Synthesis of nonlabeled 16α-iodo-oestradiol

16α-Bromo-3-methoxyoestra-1,3,5(10)-triene-17-one:
This compound, m.p. 175–177 °C, was prepared by bromination of estrone methylether with cupric bromide.

16β-Bromo-3-methoxyoestra-1,3,5(10)-triene-3,17β-diol:
This compound, m.p. 113–115 °C, was prepared by epimerization of the preceding compound with potassium bromide in dimethylformamide, followed by reduction with sodium borohydride in methanol (15).

16β-Bromooestra-1,3,5(10)-triene-3,17β-diol (16β-bromo-oestradiol):
Prepared by the cleavage of the methyl ether with 45% hydrogen bromide in acetic acid following the procedure in l.c. (16); m.p. 178–180 °C and 254–255 °C. It crystallizes with 0.5 mol methanol.

16α-Iodo-oestra-1,3,5(10)-triene-3,17β-diol (16α-iodo-oestradiol):
16β-Bromooestra-1,3,5(10)-triene-3,17β-diol (0.735 g, 2 mmol) and sodium iodide (0.33 g, 2.2 mmol) were dissolved in methyl ethyl ketone (10 ml) and the reaction mixture was heated at 70 °C overnight. The liquid was poured into water (150 ml) and extracted with methylene chloride (3 × 30 ml). The extracts were successively washed with diluted sodium thiosulphate and water, dried with sodium sulphate and evaporated. The residue was recrystallized from ethanol, m.p. 190–192 °C (11).

Competition assay

Competitors

The following compounds were investigated for their relative binding affinities for the human uterine oestrogen receptor: oestradiol, diethylstilboestrol, 16β-bromo-oestradiol, 16α-iodo-oestradiol. The tubes were prepared as follows: To each tube 50 µl of [^{3}H]oestradiol (in assay buffer) was pipetted to give a final concentration of 8 nmol/l. Then aliquots of 50 µl containing the various competitors (in assay buffer) at 6 different concentrations (range 0.1 nmol/l–10 µmol/l) were added. Finally, to each tube 100 µl of human uterine cytosol were added and after gentle shaking the tubes were incubated overnight at 4 °C. The reactions were terminated by the addition of 0.5 ml dextran coated charcoal-suspension. After 10 min of incubation under gentle shaking the tubes were centrifuged for 10 min at 1500 *g*. 0.5 ml of the supernatant were transferred to scintillation vials and after addition of 10 ml scintillation cocktail they were counted for radioactivity. All determinations were carried out in triplicate.

Binding to human sex hormone binding globulin (SHGB)

Human pregnancy serum was stripped with charcoal of endogenous steroids. The serum was used in a dilution with assay buffer of 1 : 10. The following compounds were investigated for their relative binding affinities: dihydrotestosterone, 3,17β-oestradiol, 16β-bromo-3,17β-oestradiol, 16α-iodo-3,17β-oestradiol. To each tube 50 µl of [^{3}H]dihydrotestosterone (in assay buffer) was pipetted to give a final concentration of 8 nmol/l. Aliquots of 50 µl containing the various competitors (in assay buffer) at 6 different concentrations (range 0.1 nmol/l–10 µmol/l) and 100 µl of diluted serum were added. After incubating for 60 min at room temperature plus 10 min in an ice-bath, 0.5 ml of dextran coated charcoal suspension were added and the tubes were treated as described above.

Rate of association

The time course of binding of [^{3}H]oestradiol as well as of [^{125}I]oestradiol to the oestrogen receptor was established.

Aliquots of human uterine cytosol (0.1 ml) were incubated with 0.1 ml aqueous solutions of labeled ligands ([^{3}H]oestradiol and [^{125}I]oestradiol, final concentrations 8 nmol/l) for various time intervals (t = 0.33, 0.66, 1, 1.5, 2, 3, 5, 8, 16, 24 h) at 4 °C (total binding). Nonspecific binding was determined by adding to another set of tubes a 200-fold excess of diethylstilboestrol. All incubations were carried out in triplicate. At the time intervals indicated, the reactions were stopped by the addition of 500 µl of dextran coated charcoal-suspension. The tubes were incubated under gentle shaking for 10 min at 4 °C followed by 10 min centrifugation at 1500 *g*. 0.5 ml aliquots of the supernatant were transferred into scintillation vials and counted for radioactivity after the addition of 10 ml of scintillation cocktail.

Dextran-coated charcoal assay

100 µl of aqueous solutions of [^{3}H]oestradiol or [^{125}I]oestradiol (final concentrations 0.25–4 nmol/l) were pipetted either alone (in duplicates, total binding) or in the presence of a 200-fold excess of diethylstilboestrol into 75 × 9 mm glass tubes. Then 100 µl of cytosol were added. The tubes were incubated overnight at

0–4 °C. To remove the unbound steroid, 500 µl of dextran-coated charcoal were added to each tube and incubated for 10 min under gentle shaking. After centrifugation, 500 µl of the supernatant were withdrawn and counted for radioactivity in a Beckman LS 7500. The liquid scintillation counter was calibrated using tritium and ^{125}I reference sources with known radioactivity (min^{-1}) values.

Protein assay

The protein concentrations of the cytosols were determined according to the method of *Waddell* (17). A BIO-RAD protein standard was used for the standard curve.

Calculation of the results

All data were expressed as disintegrations per minute (min^{-1}) and were transformed according to the method of *Scatchard* (18). For this transformation the computer program of *Schwarz* (19) and a HP 97 calculator (Hewlett Packard) was used. For each titration curve the K_d-value, correlation coefficient (by linear regression), intercept on the abscissa (equal to the molar concentration of binding sites) and the oestradiol receptor concentrations (fmol/mg cytosol protein) were calculated.

Results and Discussion

The new ligand 16α-iodo-3,17β-oestradiol has to be carefully characterized prior to use for routine oestradiol receptor assay. It provides several advantages compared to [^{3}H]oestradiol such as higher specific activity (12), less problems with radioactive waste and the possibility for simultaneous determination of oestrogen and progesterone receptors by a double labeling assay using [^{125}I]oestradiol and [^{3}H]promegestone (R 5020) (20).

The binding site on the oestrogen receptor hardly tolerates modifications of the oestradiol molecule without significant loss of binding affinity. Introduction of such a bulky atom like iodine on the 16α-position does not seem to alter the binding affinity to the oestradiol receptor. Figure 1 shows a representative specificity experiment comparing several oestrogen derivatives and diethylstilboestrol. The relative binding affinities, determined according to *Korenman* (23), of oestradiol and 16α-iodo-oestradiol are identical (tab. 1).

Tab. 1. Relative binding affinities (RBA) of various competitors for human oestrogen receptor and human sex hormone binding globulin

Human oestrogen receptor		Human sex hormone binding globulin	
Competitor	RBA	Competitor	RBA
oestradiol	1.0	dihydrotestosterone	1.0
diethylstilboestrol	1.2	oestradiol	0.7
16β-bromo-oestradiol	0.01	16β-bromo-oestradiol	0.68
16α-iodo-oestradiol	1.0	16α-iodo-oestradiol	0.01

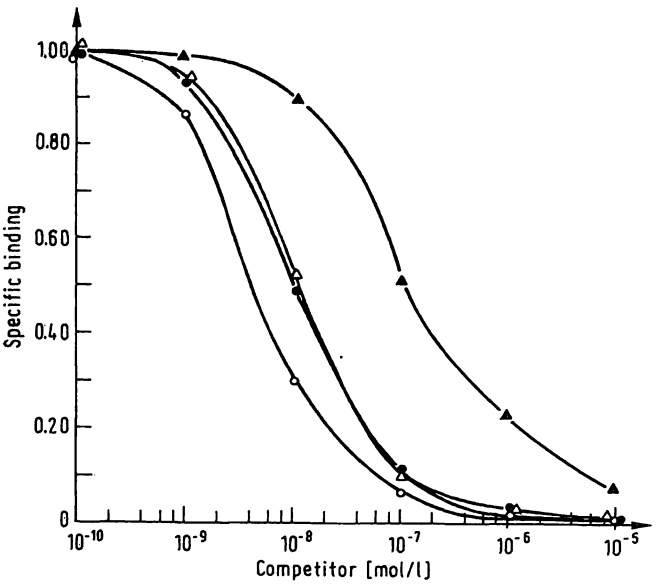

Fig. 1. Relative binding affinities for various oestrogens for human uterine oestrogen receptor.

The following compounds were investigated for their relative binding affinities: oestradiol (●), diethylstilboestrol (○), 16β-bromo-oestradiol (▲) and 16α-iodo-oestradiol (△). Aliquots of human uterine cytosol were incubated with [^{3}H]oestradiol (final concentration 8 nmol/l) and with increasing concentrations (range 0.1 nmol/l–10 µmol/l) of the various competitors at 4 °C overnight. The unbound steroid was removed with dextran-coated charcoal.

One of the intermediate products of synthesis is the 16β-bromo derivative of oestradiol. The binding site of the oestradiol receptor does not allow a modification in 16β and the relative binding affinity is decreased to 0.01. Diethylstilboestrol, used for determination of nonspecific binding shows a relative binding affinity of 1.2. Binding to SHBG results in a completely different situation (fig. 2). Compared to dihydrotestosterone with an relative binding affinity of 1.0, oestradiol shows less binding affinity to SHBG with a relative binding affinity of 0.7. The 16β-bromo-oestradiol resembles oestradiol closely in its affinity (0.68). Very interestingly, SHBG tolerates modifications in the 16β-position. The 16α-iodo-oestradiol on the other hand does not bind at all to this serum protein. Similar results are obtained using [^{125}I]oestradiol. The time course of binding of [^{125}I]oestradiol to oestradiol receptor is different from that of [^{3}H]oestradiol. The bindings of [^{3}H]oestradiol reaches equilibrium at 4 °C after 3 h, whereas [^{125}I]oestradiol reacts much more slowly, i.e. equilibrium is reached at 4 °C after 9 h (fig. 3), which is similar to the findings of *Thibodeau* (14). *Pieslor* et al. (21) have reported incubation times of 4 h only, which is not sufficient. It seems that the iodine alters not the affinity, but the kinetics of binding to the oestradiol receptor.

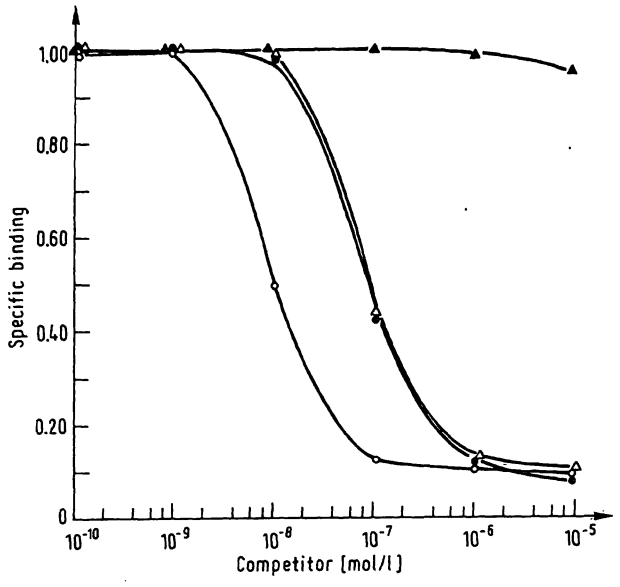

Fig. 2. Relative binding affinities for various oestrogens to human sex hormone binding globulin (SHBG).
The following compounds were investigated for their relative binding affinities: dihydrotestosterone (○), oestradiol (●), 16β-bromo-oestradiol (△) and 16α-iodo-oestradiol (▲). Aliquots of human pregnancy serum, stripped of endogenous steroids with dextran-coated charcoal, were incubated with [^{3}H]dihydrotestosterone (final concentration tion 8 nmol/l) and increasing concentrations (range 0.1 nmol/l—10 µmol/l) of the various competitors for 60 min at room temperature. The tubes were then kept in an ice-bath for another 10 min prior to removal of the unbound steroid with dextran-coated charcoal.

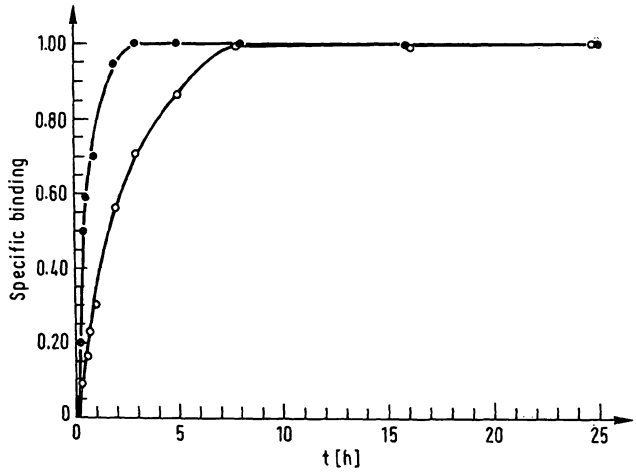

Fig. 3. Time course of binding of [^{3}H]oestradiol (●—●) and [^{125}I]oestradiol (○—○) to human uterine oestrogen receptor.
Aliquots of human uterine cytosol (0.1 ml) were incubated with 0.1 ml aqueous solutions of labeled ligands ([^{3}H]oestradiol and [^{125}I]oestradiol, final concentrations 8 nmol/l) for various time intervals (t = 0.33, 0.66, 1, 1.5, 2, 3, 5, 8, 16, 24 h) at 4 °C (total binding). Nonspecific binding was determined by adding to another set of tubes a 200-fold excess of diethylstilboestrol. All incubations were carried out in triplicate. At the time intervals indicated, the reactions were stopped by the addition of 500 µl of dextran coated charcoal suspension. The tubes were incubated under gentle shaking for 10 min at 4 °C followed by 10 min centrifugation at 1500 *g*. 0.5 ml aliquots of the supernatant were transferred into scintillation vials; after the addition of 10 ml of scintillation cocktail, these were counted for radioactivity.

100 human mammary tumour cytosols were investigated for oestradiol receptor concentrations with both ligands, [^{125}I]oestradiol and [^{3}H]oestradiol respectively (fig. 4). 20 of these contained oestradiol receptor levels below 5 fmol/mg protein, which was taken as the detection limit. Taking 10 fmol/mg protein as the clinical cut off level for being oestradiol receptor positive, one out of 100 was found to be false positive and 2 out of 100 false negative, when the [^{3}H]oestradiol assay was used.

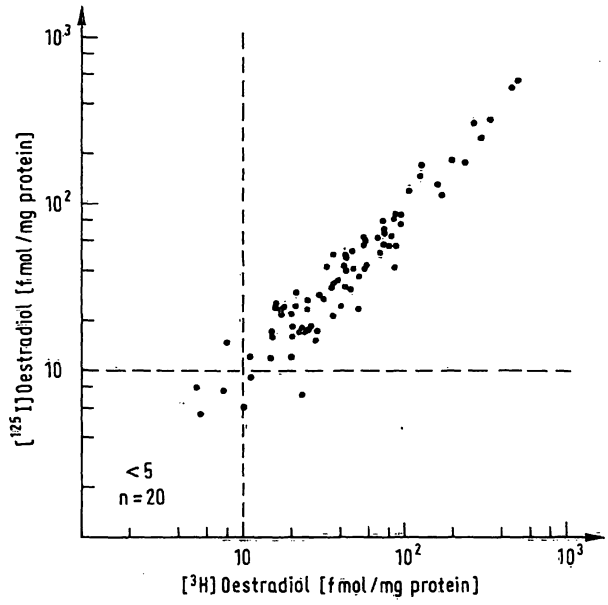

Fig. 4. Correlation of oestrogen receptor concentrations in 100 human mammary tumour cytosols using either [^{3}H]oestradiol or [^{125}I]oestradiol.

For comparison of the effectiveness of the two ligands the following statistical calculations were performed:

1. determination of the linearity of the correlation of the receptor concentrations obtained with the two ligands
2. calculation of the linear regression.

If one assumes an equivalency of the two ligands the slope of the regression should be 45°. The factor of proportionality would then be 1.0 and the regression line should pass through the origin. This would further imply that the coefficient of regression a is only randomly different from 0 and the coefficient of regression b is only randomly different from 1.0. To check this assumption a joint confidence region for the parameters a and b of the regression line was calculated according to *Brownlee* (22).

For the linear regression (independent variable [^{3}H]oestradiol, dependent variable [^{125}I]oestradiol) the following sample estimates were obtained: n = 100, r = 0.982, $y_i = 3.04 + 1.00035\,x$. For a

0.95 probability of confidence it can be stated that the sample estimates are indeed comparable according to the criteria of equivalency of the two ligands.

The mean K_d-values obtained were 0.5 ± 0.3 nmol/l using [^{3}H]oestradiol and 0.42 ± 0.25 nmol/l for [^{125}I]oestradiol. No statistical difference between the obtained K_d-values for $\alpha \leq 0.1$ could be detected.

Intra- and interassay variance

Intraassay variance

A batch of human uterine cytosol was prepared and assayed ten times for oestradiol receptor concentration using either [^{3}H]oestradiol or [^{125}I]oestradiol. The mean of oestradiol receptor concentrations obtained by *Scatchard* plot analysis was 52.18 fmol/mg protein with a standard deviation of ± 4.4. Using [^{125}I]oestradiol the mean of the oestradiol receptor determination was 54.5 ± 4.0 fmol/mg protein. An unpaired t-test for $\alpha \leq 0.1$ did not show any differences.

Interassay variance

A control powder was assayed 13 times for oestradiol receptor concentrations using [^{3}H]oestradiol as well as [^{125}I]oestradiol. The control powder contained 76 ± 24 fmol/mg protein as determined by the manufacturer. Using [^{3}H]oestradiol a mean oestradiol concentration of 81 ± 19 fmol/mg protein was found, while [^{125}I]oestradiol gave a value of 88 ± 22 fmol/mg protein. These results were compared statistically with each other as well as with the concentrations determined by the manufacturer. Statistical analysis of the three tests did not reveal a difference, with $\alpha \leq 0.01$.

In conclusion, [^{125}I]oestradiol proves to be a suitable ligand for oestradiol receptor determinations with several advantages compared to [^{3}H]oestradiol, such as higher specific activity, no binding to serum proteins, minor problems with radioactive waste and the possibility of using it in a double labeling assay for simultaneous determination of oestradiol and progesterone receptors, together with [^{3}H]promegestone (R 5020).

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